STUDIES OF ANTIBIOTIC RESISTANT MUTANTS OF *BACTEROIDES FRAGILIS* OBTAINED BY Cs-137 IONIZING RADIATION

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To the Provost of the Graduate School:

I am submitting herewith a dissertation written by Ali Owsat Azghani entitled "Studies of antibiotic resistant mutants of Bacteroides fragilis obtained by Cs-137 ionizing radiation." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Radiation Biology.

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Ali Owsat Azghani

STUDIES OF ANTIBIOTIC RESISTANT MUTANTS OF *BACTEROIDES FRAGILIS* OBTAINED BY Cs-137 IONIZING RADIATION

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The genus *Bacteroides* is an obligate anaerobic bacillus normally found in the upper respiratory tract, the colon, and the genitourinary system. The project reported here was undertaken because of the high frequency of hospital infections attributed to *B. fragilis*, and the increased resistance of the bacteria to commonly used antibiotics.

Cs-137 gamma irradiation was used to induce antibiotic resistant mutants in *B. fragilis* in the presence of *Escherichia coli* B/r membrane fragments, employed as reducing agent. Based on a dose-survival curve, an effective radiation dose of 1.54×10^4R (3.99 C/Kg) was used to induce mutations to rifampicin and tetracycline resistance in the test organism. The antibiotic resistant mutants of *B. fragilis* were utilized to reveal the mechanism by which this group of organisms becomes resistant to select chemotherapeutic agents.

Studies on tetracycline resistant mutants of *B. fragilis* isolated after irradiation, suggest that the

resistance to this antibiotic is associated with the outer membrane permeability. The difference in inhibitory action of rifampicin on RNA polymerase activity, from rifampicin sensitive and resistant strains of B. fragilis, reveals that this enzyme is a possible suitable target for inhibition of bacterial growth in anaerobes by rifampicin.

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INTRODUCTION

The importance of Gram-negative obligate anaerobic bacteria, especially Bacteroides, has been recognized during the past few years. The growing interest in genetic studies of Bacteroides is due partly to the involvement of this organism in disease and the emergence of antibiotic resistant strains. This creates great concern, because these Gram-negative anaerobes are the major bacterial species in the human intestine and may form a significant reservoir of antibiotic resistant genes in the gut. Investigations of mechanisms of resistance to antibiotics in Bacteroides may reveal new knowledge that could apply to a better understanding of the use of chemotherapeutic agents in resistant infections of the respiratory system and diseases affecting the blood.

The literature concerned with anaerobic bacteria of the genus Bacteroides does not contain any reports on the use of ionizing radiation for induction of mutations. Mutation studies of anaerobes present additional technical problems to the investigator besides the essential anaerobic growth requirements. These difficulties had to be overcome by using less complicated techniques for

irradiation of B. fragilis, and for isolation of mutants of this organism.

The purpose of the investigation was to produce mutants by the use of Cs-137 ionizing radiation and to study the properties of resultant isolated antibiotic resistant strains. The major objectives were to: 1) establish methods of growing the anaerobe Bacteroides fragilis under optimum conditions, 2) produce mutants by the use of Cs-137 ionizing radiation, 3) isolate biochemical and antibiotic resistant mutants and to grow and identify their growth requirements and antibiotic sensitivities, 4) establish the mode of action of antibiotics and the mechanisms of antibiotic resistance of the B. fragilis mutants, and 5) determine biochemical characteristics and reactions of the strains to other antibiotics.

LITERATURE REVIEW

Exposure to ionizing radiation is known to produce deleterious effects in all forms of life (Bacq and Alexander 1961; Arena 1971). Gamma radiation consists of electromagnetic rays with high penetration power and a long primary path. These photons transfer their energy to the media through which they travel, primarily by 1) the formation of ion pairs and 2) by excitation of atoms. Ionization seems to be the more significant of the two events producing biological changes (Bacq and Alexander 1961; Arena 1971).

The importance of DNA as a primary target for radiation induced inactivation of cells is well established (Ginoza 1967; Alper 1979). Ionizing radiation can damage DNA bases. Exposure to radiation can break either one or both of the double helix DNA strands. If both strands of the helix are broken less than five nucleotides apart, degradation of the DNA molecules can occur. Breaks of single strand are usually efficiently repaired (Norton 1981). During this repair process, mutations may result due to faulty replication of the DNA at the damaged site (Arena 1971; Dertinger and Jung 1970).

Alper (1968) proposed a second important site, involving DNA-membrane complexes, for damage by ionizing radiation. In contrast to isolated DNA, such complexes obtained from Escherichia coli B/r have been shown to exhibit enhanced radiosensitivity in aerated suspensions (Cramp et al. 1972). Results of studies by Redpath and Patterson (1978) showed that radiosensitivity of E. coli increases with the number of carbon to carbon double bonds in the chains of fatty acids used in the medium in which the organism is grown. These authors suggested that a post irradiation peroxidation process could be the primary mutation mechanism in the observed radiosensitivity, and the membrane region provides a potentially important site for radiation damage leading to cell death.

The survival of B. fragilis after inactivation by ultraviolet (UV) radiation has been studied (Jones et al. 1980; Webb and Lorenz 1970; and Slade et al. 1981). It was found that B. fragilis is more sensitive to far-UV radiation (245 nm) in the presence of oxygen than under anaerobic conditions (Jones et al. 1980). For other aerobic and facultative anaerobic bacteria it has been shown that inactivation by far-UV light is independent of the presence of oxygen (Webb and Lorenz 1970). To determine whether this phenomenon was due to oxygen

radicals or peroxides, Slade et al. (1981) utilized quenchers such as catalase, or superoxide dismutase, and concluded that the cells were more sensitive to UV irradiation in the presence of oxygen than to oxygen after irradiation.

Far-UV induced repair systems in the obligate anaerobe B. fragilis differ from those in E. coli. Schumann et al. (1982) showed that exposure of B. fragilis cells to far-UV light resulted in the induction of a new protein and the increased synthesis of two proteins which were normally produced in small amounts in unirradiated cells. The molecular weights of these proteins from far-UV light exposed B. fragilis were different from those obtained from E. coli comparably treated. Furthermore, the induction of the three inducible proteins in B. fragilis was affected by caffeine (Schumann et al. 1982) which is known to inhibit the excision repair and host reactivation in E. coli. Conversely, sodium arsenate which inhibits recA-dependent repair in E. coli, did not affect induction of the proteins in B. fragilis. The differences reported by Schumann et al. (1982) indicate that the far-UV induced repair systems in B. fragilis are not the same as those in E. coli, which is generally accepted as the model for a bacterial repair system.

The fact that "animalcules" can develop without air was demonstrated for the first time by Leeuwenhoek in 1680 (Dobell 1960). The terms aerobies and anaerobies were introduced by Pasteur in 1861, who described butyric fermentation due to an anaerobic sporeforming bacillus, now known as Clostridium butyricum (Sonnenwirth 1972). Considerable effort has since been expended in the search for the cause of oxygen toxicity in anaerobes (O'Brien and Morris 1971). Much earlier McLeod and Gordon (1923) suggested that inhibitory concentrations of hydrogen peroxide accumulate when catalase-negative anaerobes are exposed to air. However, Mateles and Zuber (1964) showed that the addition of crystalline catalase to aerated cultures of Clostridium exhibited little growth stimulation effects. This study, however, did not rule out the action of organic peroxides or similar compounds that accumulate in the oxidized medium.

The influence of oxygen on enzymatic activities of Clostridium acetobutylicum was examined by O'Brien and Morris (1971). They found that oxygen increased the enzymatic activity of nicotinamide adenine dinucleotide oxidase (NADH oxidase). O'Brien and Morris (1971) speculated that at high concentrations of oxygen the cells were deprived of NADH and therefore biosynthetic functions

were interrupted. It was also found that specific activities of a number of enzymes of the glycolytic and butyrate-producing pathways tested by these investigators were not affected by oxygen.

The oxidation reduction potential (E_h) is another factor affecting the growth of anaerobic bacteria (Hentges and Maier 1972). A limiting E_h value of 230 - 250 mV at pH 6 was obtained by Barnes and Ingram (1956) for Clostridium perfringens. They found that at an E_h of -45 mV there was hardly any growth lag, but as the redox potential was increased, the lag phase became longer. At a reduction potential of about 230 mV Clostridium perfringens was totally destroyed. Using potassium ferrocyanide as an artificial electron acceptor, O'Brien and Morris (1971) showed that C. acetobutylicum could be maintained in an anaerobic condition at an E_h value of 370. C. acetobutylicum could not tolerate an oxygen tension of 40 µm in a culture medium with an E_h of -50 mV, indicating that the free 0_2 in the medium, rather than E_h, was the crucial factor in causing growth inhibition.

To improve cell multiplication of anaerobic bacteria in broth cultures, Holdman and Moore (1972) introduced a technique known as the VPI (Virginia Polytechnic Institute) method. It is based on the use of pre-reduced

anaerobically sterilized medium. Each culture tube becomes its own anaerobic chamber, involving a "gas out" procedure during inoculation and the use of a tight stopper on the tube during the incubation period.

An inovative method used to grow anaerobic bacteria was introduced by Adler and Crow (1981). They added partially purified membrane fractions, obtained from E. coli B/r, to the culture to remove oxygen from the bacteriological medium. This oxygen reduction occurs because of the presence of an active cytochrome electron transport system located in the cytoplasmic membrane (Adler et al. 1981). The membrane fractions are effective when used either in broth or in solid media. Small quantities of active membrane fractions in the medium produce anaerobic conditions for growth and maintain this state even if more air is reintroduced. This additive makes it unnecessary to boil the medium before use to drive off the oxygen or sparge the culture tubes with oxygen free gas during cultivation (Adler and Crow 1981).

Subspecies of B. fragilis distasonis, B. fragilis fragilis, B. fragilis vulgatus, B. fragilis oratus, and B. fragilis thetaiotaomicron are recognized as important pathogens in suppurative diseases and other infections. Of this group, B. fragilis subsp. fragilis is the most

significant. It is being isolated with the highest frequency from serious infections and is the most common anaerobic bacterial species that invades the blood stream (Tally et al. 1983).

Chemotherapy of infections due to Bacteroides is a serious problem, since most strains are resistant to many antibiotics, including aminoglycoside and beta-lactam drugs. Furthermore, the incidence of antibiotic resistant strains seems to be increasing (Rashtchian et al. 1982). It has been reported that 50 to 60% of Bacteroides species isolated in the United States, and 80.5% of those found in France, were resistant to tetracycline (Overman et al. 1974; Privitera et al. 1981).

Tetracycline antibiotics used in therapy include the biosynthetic substances tetracycline, oxytetracycline, and chlorotetracycline all derived from Streptomyces and several modified derivatives such as demethylchlorotetracycline, doxycycline, minocycline, methacycline, and pyrrolidinemethyltetracycline. All tetracyclines have a broad antimicrobial spectrum and are bacteriostatic at low and bactericidal at high concentrations (Kurytowicz 1976). Tetracycline inhibits protein synthesis by interfering with the binding of aminoacyl-transfer

ribonucleic acid to the ribosomal A site in E. coli (Suarez and Nathans 1965).

Most bacteria show cross-resistance to tetracycline, oxytetracycline, and chlorotetracycline, but those resistant to these antibiotics may also be sensitive to doxycycline and minocycline. This characteristic could be due to the fact that modified tetracyclines are more lipophilic (Kurytowicz 1976). As a result, they penetrate across biological barriers more readily and are less prone to form chemical complexes with calcium and phosphate ions that are less soluble (Kurytowicz 1976).

Tetracycline resistance as a therapeutic problem was first recognized to be world wide in scope during the dysentery epidemic in Japan in the mid-1950s (Akiba et al. 1960). Tetracycline resistance also was demonstrated in many other bacterial organisms, including Bacteroides (Marcini and Behme 1977; Privitera et al. 1979). Emergence and propagation of antibiotic resistance were the result of selection of those cells that are not destroyed by the drug, as well as the widespread use of these potentially antibacterial chemotherapeutic agents in infections of man and animals throughout the world (Levy 1984).

Transfer of tetracycline resistance has been demonstrated between species of Bacteroides (Privitera et al. 1979; Rashtchian et al. 1982) and from B. fragilis to E. coli (Mancini and Behme 1977). In at least some strains such as B. fragilis, both transfer and expression of tetracycline resistance markers were enhanced in the presence of sub-inhibitory concentration of tetracycline (Rashtchian et al. 1982).

Studies of tetracycline metabolism demonstrated both active and passive uptake of the antibiotic into sensitive cells (McMurry and Levy 1978). Work by Arima and Izaki (1963) established that tetracycline actively accumulated in E. coli, a finding that was confirmed by many investigators (Franklin and Godfrey 1965; McMurry and Levy 1978). Studies by Murry and Levy (1978) revealed an early, rapid, and energy-independent transport system for this antibiotic in E. coli cells. The energy-dependent uptake of the antibiotic starts usually after 6 min incubation of the cells with tetracycline.

Some strains of Bacteroides, like many other bacteria, contain plasmids, also called extrachromosomal genetic elements. Plasmids are nonessential for the growth of bacterial cells, so that under most conditions they may be gained or lost without lethal effects

(Clowes 1972). Plasmids are known to be responsible for many bacterial characteristics of both clinical and ecological significance. Such properties include resistance to antibiotics (Helinski 1973), toxin production including hemolysin (Jacob et al. 1975), and hydrocarbon metabolism (Chakrabarty et al. 1973). Plasmids are also known to confer conjugal ability to the cell in which they reside (Tinnell and Macrina 1976).

The molecular basis of antibiotic resistance in bacteria containing plasmids has been elucidated (Bryan and Parr 1984). Plasmids may encode an enzyme that alters the target site for an antibiotic, consequently reducing the binding of the antibiotic to the target. The plasmid may carry the genes for an insensitive target to be replaced by the original sensitive one. Detoxification of antibiotics by enzymes encoded on plamid DNA is another mechanism by which bacteria become resistant to antibiotics. A well known example is β-lactamase enzyme production. This enzyme hydrolyzes the β-lactam ring of penicillin and cephalosporins. Finally, a plasmid-encoded system alters the permeability of the membrane so that no antibiotic is accumulated inside the cell (Davies and Kagan 1977).

Guiney and Davis (1978) identified a conjugative plasmid in B. orchraceus 2228 which specifies resistance to chloramphenicol, tetracycline, kanamycin, and streptomycin. The plasmid was transferred by conjugation to E. coli and was maintained in this new host. This observation strongly suggests that plasmid transfer may occur in vivo between Bacteroides and wild E. coli strains, enabling Bacteroides to form a significant reservoir of antibiotic resistant genes in the gut. Results reported by Privitera et al. (1979) indicated that resistance to clindamycin, erythromycin, and streptogramins, were transferable from a clinical isolate of B. fragilis subsp. fragilis to the sensitive strain of B. fragilis subsp. fragilis and that the minimal inhibitory concentrations (MICs) of the antibiotics for the recipient were similar to those of the donors. Privitera et al. (1979) suggested that the mechanism of transfer could be conjugation because they were unable to observe transformation and transduction. Interspecies transfer in B. fragilis of clindamycin and erythromycin resistance from subsp. fragilis to thetaiotaomicron has been demonstrated by Tally et al. (1979).

The genetic basis of the resistance of B. fragilis 12256 to penicillin G, tetracycline, and clindamycin was

studied by Rashtchian et al. (1981). They found two species of plasmids with molecular weights of 3.4 and 1.95 million daltons (Mdal) which were not self transmissible to E. coli by conjugal transfer experiments, probably due to their molecular weight. Transformation experiments demonstrated that the 1.95 Mdal plasmid controlled resistance to penicillin G and tetracycline. In addition, Rashtchian et al. (1981) observed that strains which were spontaneously cured of the plasmid, reverted to penicillin G and tetracycline sensitivity.

Rifampicin, a chemically modified natural rifamycin has found the widest therapeutic applications among the rifampicin antibiotics (Kurytowicz 1976). This antibacterial agent specifically inhibits DNA dependent RNA-polymerase activity in E. coli (Hartmann et al. 1967).

RNA polymerase contains four different subunits, designated α, β, β and σ (Lehninger 1975). Of these subunits β is involved in nucleoside triphosphate binding and responsible for the interaction with the initiation inhibitor rifampicin (Gado et al. 1982).

MATERIALS AND METHODS

The bacterial strain

Bacteroides fragilis VPI 531 was obtained from the Anaerobic Laboratory, Virginia Polytechnic Institute (Blacksburg, VA), and maintained in pre-reduced chopped meat broth. The identity of the strain was confirmed by the methods outlined in the fourth edition of the Anaerobe Laboratory Manual of the Virginia Polytechnic Institute (Holdman et al. 1977).

Cultures for daily experiments were grown in Schaedler broth (Difco), Brain Heart Infusion (Gibco) complemented with hemin (Sigma) and cysteine hydrochloride (Nutritional Biochemical Corp.). Antibiotic medium No. 3 (Gibco) supplemented with hemin was used for the antibiotic sensitivity tests. Chopped meat medium was prepared either from lean beef according to the procedure by Holdman et al. (1977), or by rehydrating Gibco Cooked Meat Medium in demineralized water containing cysteine hydrochloride (0.5 mg/ml). All media were sterilized in an autoclave at 121 C, 18 psi for 10 min.

Anaerobic procedures

The plate cultures were incubated anaerobically at 37 C for 24 h in polycarbonate plastic GasPak jars by

either one of two methods. In the evacuation-replacement method, after sealing the jar, air was removed by vacuum. The empty jar was then filled with a gas mixture of 80% N_2, 10% H_2, 10% CO_2 (Liquid Air Corp.). The process of evacuation-replacement was repeated three times. In the second method, 10 ml water was added into a commercially available H_2 and CO_2 generator envelope (BBL) (Figure 1). The hydrogen was necessary to reduce the oxygen in the presence of fresh palladium-coated alumina pellets as the catalyst. The CO_2 was stimulatory for the growth of the organisms. Anaerobic conditions, as indicated by methylene blue strip indicators (BBL), were obtained in one to two hours by using the GasPak method.

To prepare pre-reduced medium the oxygen was driven off by boiling, then cysteine was added for further reduction of the ingredients. Oxygen was kept away from the medium by flushing with oxygen-free gas during manipulations (Figure 1), and by using tightly-stoppered tubes while inoculating or storing the medium. Resazurin (0.4%) was utilized in the medium as E_h indicator.

Aerobic techniques for growing anaerobes

Membrane fractions derived from Escherichia coli B/r have been shown to remove oxygen from a variety of bacterial media. The membrane fractions were prepared by

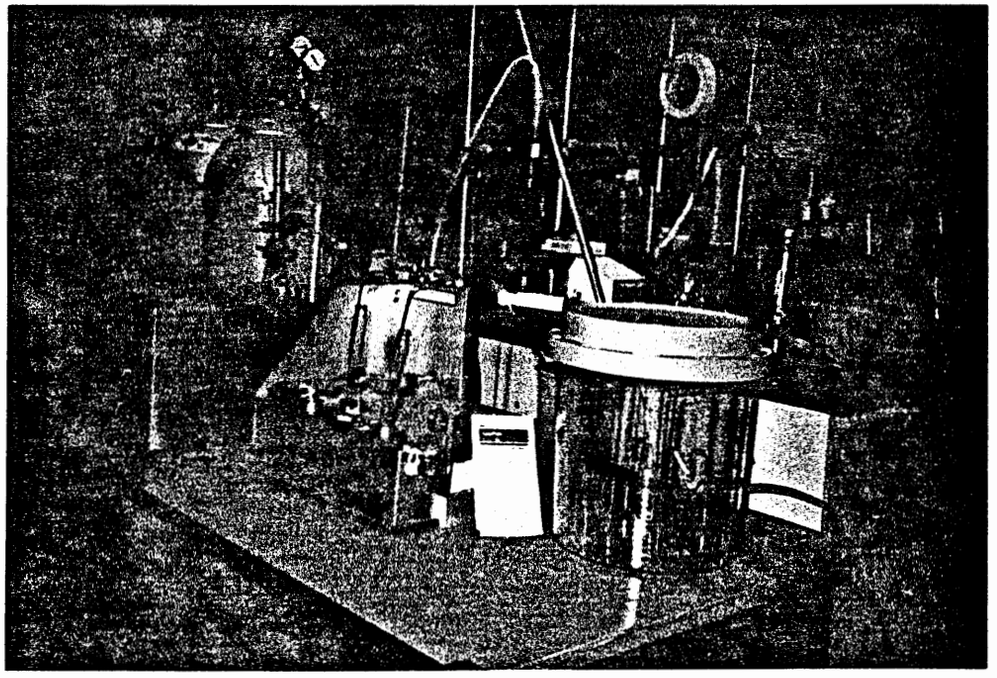

Figure 1--From left to right: Oxygen free gas tank, VPI anaerobic culture inoculation system (Bellco, Inc.), anaerobic GasPak package, anaerobic GasPak jar.

the method of Adler et al. (1981). E. coli B/r cells were grown to stationary phase in nutrient broth supplemented with yeast extract and glucose. The cells were centrifuged for 10 min at 10,000 rpm, washed with 0.01 M cold HEPES buffer, pH 7.5 (N-2-hydroxy-ethylpiperazine-N'-2-ethane-sulfonic acid. A cell pellet of 10 g was suspended in 25 ml of HEPES buffer, centrifuged for 10 min at 10,000 rmp at 0 C, and then resuspended in 50 ml of HEPES buffer. The suspension was passed through a French Press (SORVALL, RIBI Cell Fractionator Model RF-1) three times at 20,000 psi, and then centrifuged at 12,000 g for 10 min at 0 C. The resulting supernate was centrifuged for 5 h at 175,000 g at 5 C. The pellet from this high speed centrifugation was resuspended in 25 ml of HEPES buffer to allow filtration through a 0.22 μm Sterivex-GS Millipore filter. Sterile membrane fragments were then frozen in aliquotes of 1 ml.

Reagents and media used

A hemin solution was prepared by dissolving 50 mg of hemin in 1 ml of 1N NaOH. Distilled water was used to bring the total volume to 100 ml. The hemin solution was autoclaved at 121 C for 10 min and stored in a brown bottle. The resazurin solution was made by dissolving 25 mg of resazurin (Sigma) in 100 ml of distilled water.

To prepare a salt solution to be used in Peptone Yeast (PY) medium, 0.2 g of $CaCl_2$ and 0.2 g of $MgSO_4$ were dissolved in 300 ml distilled water. Water was added to bring the final volumn to 500 ml. While swirling, K_2HPO_4 (1 g), KH_2PO_4 (1 g), $NaHCO_3$ (10 g), and NaCl (10 g), were added slowly. After the salts were dissolved, 200 ml distilled water was added and the mixture was stored at 4 C.

To prepare chopped meat medium 500 g fat free lean beef was ground and mixed with 1,000 ml distilled water and 25 ml of 1 N NaOH. After the fat layer had been removed, the mixture was boiled, cooled to room temperature and filtered. Distilled water was added to the filtrate to bring the volume up to 1 liter, followed by the addition of trypticase 30 g, yeast extract 5 g, KH_2PO_4 5 g, and resazurin solution 4 ml. After boiling and cooling of the mixture, 0.5 g cysteine-HCl along with 10 ml hemin solution were added. After adjusting the pH (7.0 ± 0.2), five parts of this broth were added to one part of the meat particles in screwcapped tubes, it was flushed with oxygen free gas mixture and then autoclaved for 10 min at 121 C at 18 psi. Dehydrated Cooked Meat medium (Gibco) was also used. Ten ml of demineralized water containing cysteine-HCl (0.5 mg/ml) was added to each tube to rehydrate 1.25 g of the dry medium.

Pre-reduced Anaerobic Brain Heart Infusion broth (ABHI) was prepared by dissolving 3.7 g of BHI (Gibco) and 0.5 g of yeast extract in 100 ml of distilled water. Resazurin (0.4 ml), cysteine-HCl (0.05 g), and hemin (1 g)

were added before the mixture was dispensed into screw-capped tubes to be autoclaved.

To test for carbohydrate fermentation, a basal medium known as peptone yeast extract was used. The following was dissolved in 100 ml of distilled water: peptone (0.5 g), trypticase (0.5 g), yeast extract (1.0 g), resazurin solution (0.4 ml), and salt solution (4.0 ml). The medium was boiled, cooled, and 0.5 g cysteine-HCl along with 1 ml hemin solution were added before autoclaving. The carbohydrates, glucose, starch, and esculin, were added to the PY medium in final concentration of 1% before boiling the ingredients.

The dehydrated Schaedler medium (BBL) contains all the ingredients that B. fragilis requires, including cystein as a reducing agent. The manufacturer's directions were followed for rehydrating.

Minimal agar medium was prepared by the method of Van Tassell and Wilkins (1978). It contained per liter; $(NH_4)_2SO_4$, 2 g; sodium citrate, 0.5 g; vitamin B_{12}, 5 µg; KH_2PO_4, 7 g; K_2HPO_4, 8 g; $MnCl_2.4H_20$, 10 mg; $MgCl_2.6H_20$, 20 mg; $FeCl_3.6H_20$, 0.3 mg; $CaCl_2.2H_20$, 30 mg; $NaHCO_3$, 4 g; cystein-HCl, 0.5 g; glucose, 10 g; agar, 20 g; hemin, 5 mg; and resazurin, 1 mg. The medium was prepared by dissolving the first seven components in 350 ml distilled

water and adjusting the final pH to 6.65. The agar, $FeCl_3.6H_20$ and $CaCl_2.2H_20$ were dissolved in 500 ml of distilled water and boiled. The two mixtures were autoclaved at 121 C for 15 min. After sterilization, the molten agar was cooled to 60 C and aseptically added to the salt containing broth. Sterile autoclaved solutions of glucose (10% wt/vol), cysteine-HCl (5% wt/vol), and sodium carbonate (10% wt/vol) were aseptically added to yield final concentrations of 1%, 0.05%, and 0.4%, respectively.

Fermentation of carbohydrates

Tubes of peptone yeast broth received different carbohydrates, either glucose 1%, starch 1%, or esculin 0.5%. Changes in pH were determined after 24 h incubation at 37 C. A pH value of 5.5 or below was an indication of acid production, whereas, a pH above 6.0 was interpreted as negative, considering that the pH of PY was 6.2 or higher. To determine the hydrolysis of starch, a few drops of Gram's iodine were added to the starch broth. Expected color changes were observed immediately. If no color was seen, starch had been hydrolyzed. A blue-black color indicated that starch was still present and the reaction was recorded as negative (Holdman et al., 1977).

Indole production

In the tube test for detection of indole production 2 ml of a culture in chopped meat broth was removed for testing. One ml of xylene was added and the mixture was shaken well. After 2 min 0.5 ml of Ehrlich's reagent was added slowly. The development of a pink ring within 10 - 15 min indicated a positive reaction, whereas, a yellow ring was recorded as negative (Holdman et al. 1977).

Preparation of concentrated antibiotic stock solutions

A concentrated solution of each antibiotic was prepared by dissolving 0.01 g of the powder in 10 ml distilled water (1,000 µg per ml) and filtering it through a 0.45 µm Nalgene filter (Sybron Corp.). Rifampicin and tetracycline were dissolved in 97% ethyl alcohol. Sterile 1 ml aliquotes of each antibiotic so prepared were maintained in sterile screwcapped tubes in the deep freeze.

Antibiotic sensitivity test

The Disk-Diffusion Method was used to test for antibiotic sensitivity of the bacteria. Antibiotic medium No. 3 (Gibco) supplemented with hemin was seeded with the standardized broth culture of the strain to be used. Antibiotic disks containing penicillin, kanamycin, vancomycin, clindamycin, lincomycin, rifampicin,

tetracycline, and erythromycin (Difco), in concentrations as shown in Table 2, were then placed on the agar surface (4 disks/Petri plate). The plates were incubated at 37 C for 25 h and the diameters of the zones of inhibition of bacterial growth were then measured.

The Tube Dilution Method also measures antibiotic sensitivity of bacteria. To determine the minimal inhibitory concentration (MIC) of antibiotics, commonly used in Bacteroides infections, twofold dilutions of antibiotics ranging from 100 to 0.39 µg/ml were made in antibiotic medium No. 3 (Table 1). The dilutions were set up in replicates of three for each antibiotic. The test organisms had been incubated anaerobically at 37 C for 24 h in Schaedler broth and standardized by adjusting the turbidity to the density of the #1 McFarland nephelometer standard. The tubes were incubated at 37 C for 24 h. The MIC of the test antibiotic was the lowest concentration of the drug that did inhibit the bacterial growth.

Irradiation studies

A Mark I (Cs-137 Gamma Irradiator, Model 30, JL Shepherd and Associates) was utilized to induce mutations in Bacteroides fragilis cells. B. fragilis was grown in pre-reduced Schaedler broth for 18 - 24 h. The cells were centrifuged for 10 min at 8,000 rpm, washed with

Table 1--Dilution procedure for all antibiotics tested for their Minimal Inhibitory Concentration (MIC) on Bacteroides fragilis.

Tube	Broth*	Dilution scheme	Standardized inoculum**	Final antibiotic conc.
	mo		ml	µg/ml
1	none	0.5 ml of antibiotic from 1:5 dilution of 1 mg/ml	0.5	100
2	0.5	0.5 ml of antibiotic from 1:5 dilution of 1 mg/ml	0.5	50
3	0.5	0.5 ml from tube 2	0.5	25
4	0.5	0.5 ml from tube 3	0.5	12.5
5	0.5	0.5 ml from tube 4	0.5	6.25
6	0.5	0.5 ml from tube 5	0.5	3.125
7	0.5	0.5 ml from tube 6	0.5	1.56
8	0.5	0.5 ml from tube 7	0.5	0.78
9	0.5	0.5 ml from tube 8	0.5	0.39
10	0.5	none	0.5	0

*Antibiotic medium No. 3.
**The inoculum was diluted to the turbidity of the #1 McFarland standard.

pre-reduced phosphate buffer (pH 6.8) and resuspended in 10 ml of phosphate buffer with 0.2 µl of E. coli B/r "cytoplasmic membrane fragments" as reducing agent (Adler et al. 1981). The cells in 10 ml were then exposed in a modified 100 ml Turner bulb to 7.75 X 10^4 R/h (19.99 C/Kg) of Cs-137 for a range of time intervals. Prior to irradiation, the air in the Turner bulb was replaced with a stream of a gas mixture (10% H_2, 10% CO_2 and 80% N_2).

To construct a dose-response curve for B. fragilis, dilutions of the irradiated cells were made and a 0.1 ml cell suspension was used to inoculate Petri plates in triplicate for each dilution tested. The cultures were placed in an anaerobic jar and incubated at 37 C. After 24 h, plate counts were made and the average number of colonies was plotted against the total dose of radiation received as a function of exposure time.

Isolation of antibiotic resistant mutants

The selection of antibiotic resistant mutants was carried out on ABHI agar containing 10 µg/ml of the test antibiotic. Aliquots of the irradiated cell suspension were inoculated into ABHI broth and incubated anaerobically at 37 C for 8 h. Pour plates were made by using ABHIA containing 10 µg/ml of test antibiotic. The plates were then incubated for 24 h at 37 C in a GasPak jar.

Mutant colonies obtained on agar plates were picked and inoculated to chopped meat broth with 10 µg/ml of test antibiotic, to be maintained for later studies.

Isolation of RNA polymerase

The extraction of RNA polymerase was performed by the method of Gross et al. (1976). One hundred ml of a late log phase culture was chilled in a dry ice methanol bath and centrifuged for 10 min at 10,000 rpm at 4 C. Subsequently, the cells were lysed by the addition of several solutions named A, B, and C. One ml of solution A [0.01 M Tris-HCl, pH 7.9, 25% (wt/vol) sucrose, 0.1 M NaCl] was applied at 0 C for 15 min. Solution B (0.25 ml) (0.3 M Tris-HCl pH 7.9, 0.1 M EDTA, together with 4 mg of fresh egg white lysozyme/ml) was added for 5 min at 0 C, followed by 1.25 ml of solution C [1.0 M NaCl, 0.02 M EDTA, pH 7.0, 0.08% (wt/vol) deoxycholate] at 10 C for 10 min.

To isolate RNA polymerase from the cell lysate, 3.5 ml of solution D [17% (wt/vol) polyethylene glycol (PEG), 0.157 M NaCl, and 0.01 M dithiothreitol (DTT) prepared just before use] was added with thorough agitation. After 10 min at 0 C the mixture was centrifuged at 7,000 rpm for 10 min. This purification step, resulted in co-precipitation of RNA polymerase and DNA. RNA

polymerase was then eluted from the PEG precipitate by adding 0.5 ml of solution E [(5% wt/vol) PEG, 2.0 M NaCl, 0.01 M Tris-HCl, pH 7.4, and freshly prepared 0.01 M DTT] to the precipitate, dispersing the pellet and centrifuging at 10,000 rpm for 10 min. The supernatant was saved for later assay.

Assay of RNA polymerase

The RNA polymerase activity was assayed employing the following mixture according to the method of Clark et al. (1977). The reagents added to ice-cold test tubes were: 0.02 ml of 0.5 M Tris-HCl, pH 7.9, 0.01 M Na_2EDTA, 0.01 M dithiothreitol, and 1.857 M KCl; 0.02 ml of bovine serum albumin (0.02 mg/ml); 0.02 ml of 2 mM GTP, CTP, and UTP; 0.02 ml of 1 mM [^{3}H] ATP (at least 10 mCi/mM); 0.05 ml of 0.75 mg calf thymus DNA/ml; and up to 0.1 ml of RNA polymerase extract to make a total volume of 0.25 ml. If less than 0.1 ml of the enzyme extract was utilized, water was added to obtain an equal total volume in all assay tubes. The reaction in each tube was terminated by adding 5 ml of ice-cold 5% trichloroacetic acid (TCA), with 0.01% sodium pyrophosphate. Each sample was then filtered through a glass fiber filter (Gelman), washed three times with TCA (5%) and incubated at 90 - 100 C for 10 min. Ten ml of Aquasol scintillation cocktail [New England Nuclear (NEN)]

was added into each vial containing the dry filter, then the radioactivity was determined with a Beckman 9,000 liquid scintillation counter. To determine the effect of rifampicin on the activity of RNA polymerase, desired concentrations of the antibiotic and the enzyme were mixed and incubated at 38 C for 10 min. The incorporation of [^{3}H] ATP was then measured according to the preceding technique.

Tetracycline uptake studies

The uptake of tetracycline was determined by the incorporation of [^{3}H] tetracycline, according to the procedure by McMurry and Levy (1978). The cells were grown at mid to late log phase at 37 C, centrifuged, and resuspended in fresh Schaedler broth. After 15 min incubation at 37 C [^{3}H], tetracycline [7-^{3}H(N), 679 Ci/mmol, NEF] was added to the culture to a final concentration of 1 µg/ml. After incubation at 37 C for 1 h, 100 µl samples were removed and diluted 1:17 in cold saline buffer and centrifuged at 4 C for 10 min to remove the cells from the free tetracycline in the media.

For measurements of radioactivity, the cell pellets were resuspended in 0.5 ml of saline buffer and dissolved in 10 ml of Aquasol scintillation cocktail. The activity of the accumulated [^{3}H] tetracycline was then counted for

1 min in a Beckman 9,000 liquid scintillation counter. The background for uptake of antibiotic tetracycline was determined by adding [^{3}H] tetracycline to the culture tubes at 0 C and the procedure was carried out without incubation of the samples at 37 C.

A modification of the method described by Izaki et al. (1966), was used to determine the effect of the presence of tetracycline on the cells ability to accumulate this antibiotic. The cells were grown at mid to late log phase at 37 C, centrifuged, and resuspended in Schaedler broth containing 0.5 µg/ml of tetracycline. After 3 h incubation at 30 C, the preceding procedure for tetracycline accumulation was followed.

Beta lactamase test

The production of β-lactamase was determined according to the procedure by Bourgault and Rosenblatt (1979). A 500 µg/ml solution of nitrocefin (a thoughtful gift of Dr. Nash, University of Texas Health Center at Tyler) was used for this experiment. A loopful of the test organism was emulsified in one drop of the nitrocefin solution on a clean glass microscope slide. A change in color from yellow to red within 15 min indicated the presence of β-lactamase.

Isolation of plasmid DNA

The following method described by Welch and Macrina (1981) is based on the preferential precipitation of the high molecular-weight chromosomal DNA by sodium dodecyl sulfate (SDS) in the presence of sodium chloride. A 6 ml overnight broth culture was harvested by centrifugation at 12,000 X g for 10 min at 4 C and the cell pellet was transferred to a 1.5 ml centrifuge tube. The pellet was suspended in 1.2 ml of 1 X Tris-Na_2 EDTA (TES) buffer. This cell suspension was pelleted in a microfuge and the washed cell pellet was resuspended in 0.25 ml of a 25% sucrose solution (in 0.05 M Tris, pH 8). The following substances were then added sequentially: RNase 6 µl (5 mg/ml in 0.24 M Tris, pH 8.0), 13 µl of EDTA (0.25M), and 20 µl of egg-white lysozyme (10 mg/ml in 0.025 M Tris, pH 8.0). This mixture was incubated for 20 min at 23 C and after 20 µl of 20% sodium dodecyl sulfate in 0.01 M Tris (pH 8) was added, incubated at 23 C until visible cell lysis (clearing) occurred. Fifty microliters of 0.5 M NaCl solution was added, and the lysate was incubated at 4 C for at least 3 h. After the sodium dodecyl sulfate high salt treatment, the lysate DNA was centrifuged at 17,000 X g for 20 min at 4 C. The pellet was gently removed by using a Pasteur pipete and discarded. Freshly

re-distilled phenol (0.3 ml) saturated with 1 X TES was added to the supernatant, and the mixture was briefly blended in a Vortex mixer. Next, 0.3 ml of chloroform-isoamyl alcohol (24:1) was added and the mixture was again blended. The top aqueous layer (0.5 ml) was removed and precipitated by adding 2 volumes of ethanol and 15 µl of 5 M NaCl. The mixture was incubated at -70 C for at least 1 h and then the precipitated DNA was pelleted by centrifugation at 5,900 X g at -20 C for 20 min. The precipitated DNA was brought into solution by the addition of 60 µl of sterile distilled water. Approximately 25 µl of this preparation enabled the easy visualization of plasmid DNA components in agarose electrophoretic gels stained with ethidium bromide.

For electrophoresis, 25 µl of this preparation was used in 0.7% agarose gel (MCB) in a vertical slab gel apparatus (BIO-RAD). The electrophoresis was done for 4 1/2 h at room temperature at 20 mA and 120 V. The gel was then stained with 1 µg/ml of ethidium bromide (Sigma). The DNA was then visualized by a short-wave UV light and photographed.

Plasmid elimination

The synthesis of possible plasmid DNA in B. fragilis was impaired by elevating the incubation temperature as

described by Carlton and Brown (1981) for other organisms. The bacterial culture was incubated at 42 C for 10 h. The cells were then transferred to fresh medium and grown further at the elevated temperature. The bacteria were then tested for the presence of plasmids by agarose gel electrophoresis.

EXPERIMENTAL RESULTS

Before exposure of Bacteroides fragilis VPI 531 to radiation from a Cs-137 source, biochemical studies and antibiotic susceptibility tests were performed to confirm the identity of the test organism. The cell morphology and colony formation of the organism were also observed, as well as other physical characteristics. The results of the biochemical reactions and responses to antibiotics of the test organism are shown in Table 2. The results confirmed that the organism tested was B. fragilis, as listed in the 4th edition of the Anaerobe Laboratory Manual (Holdman et al. 1977).

According to Welch et al. (1979) B. fragilis VPI 531 contains two plasmids with molecular sizes of 2.5 and 6.8 Mdal. Plasmids are extrachromosomal DNA, which control many important bacterial properties including resistance to antimicrobial agents (Clowes 1972). The functions of the B. fragilis plasmids as found by Welch et al. (1979) are not clear. Attempts were made to isolate plasmid DNA from B. fragilis VPI 531. Strains of E. coli harboring plasmids with known molecular weights were used as controls. Figure 2 shows the electrophoretic banding patterns of the

Table 2--Biochemical characteristics and antibiotic susceptibilies of Bacteroides fragilis VPI 531 used for conformation of the organism's identity.

Tests	Reaction
Fermentation of:	
Glucose	+
Esculin	-
Starch	+
Hydrolysis of starch	+
Production of indole	-
Sensitivity to:	
Clindamycin (2 µg)	s
Erythromycin (15 µg)	s
Ampicillin (10 µg)	r
Rifampicin (30 µg)	s
Tetracycline (30 µg)	s
Penicillin (10 units)	r

+ = Positive or Present
- = Negative or Absent
s = Sensitive
r = Resistant

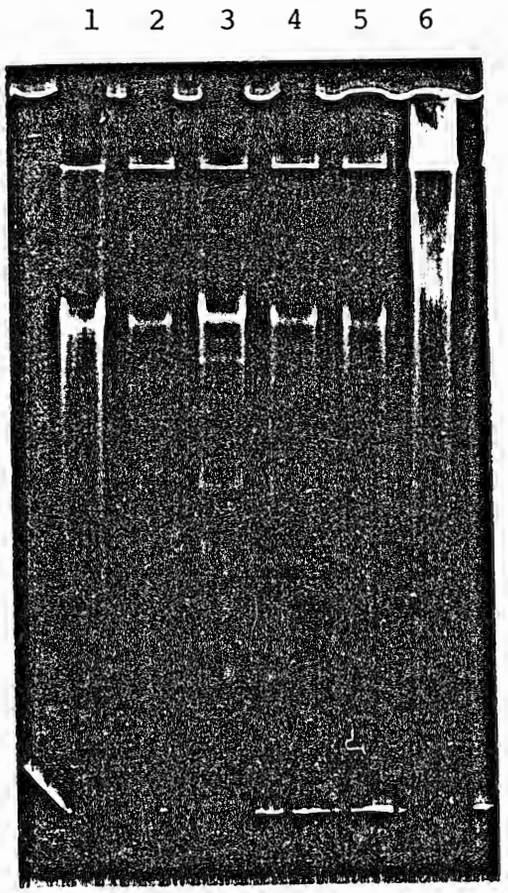

Figure 2--Agarose gel electrophoresis of ethanol-precipitated DNA from clear lysates of cells. Electrophoresis was performed in a 0.7% agarose gel at 120 V, 20 mA, for 4 1/2 h. (1 and 6) standard chromosomal DNA. (2 and 5) *B. fragilis* before and after treatment with elevated temperature. (3 and 4) molecular weight standard plasmids of *E. coli*.

cleared lysate from E. coli and B. fragilis strains. Column 2 of Figure 2 indicated that B. fragilis did not harbor any detectable plasmid DNA. Nevertheless, the organism was eliminated from possible extrachromosomal DNA (Figure 2, column 5) in order to exclude the involvement of plasmid DNA in this study. Columns 3 and 4 of Figure 2 showed the bands of plasmids with known molecular weights of 2.45 and 3 Mdal, isolated from E. coli strains. Columns 1 and 6 disclosed chromosomal DNA fragments used as controls.

B. fragilis was grown in PYG broth at 37 C for various time periods, in order to construct a growth curve. An inoculum of 0.05 ml from a 24 h culture of B. fragilis was transferred anaerobically into each tube containing 5 ml of pre-reduced PYG broth. At different times up to 9 h, after anaerobic incubation at 37 C, turbidities of the cultures were determined in a Spectronic 20 Spectrophotometer. The data obtained are shown in Figure 3. After a 2 h lag phase the logarithmic growth phase lasted for 4 h 40 min.

Effects of ionizing radiation on the survival of B. fragilis were determined. Late log phase cultures of B. fragilis were exposed to 1.29×10^3 R/min (0.33 C/Kg) of Cs-137 radiation in modified Turner bulbs for several

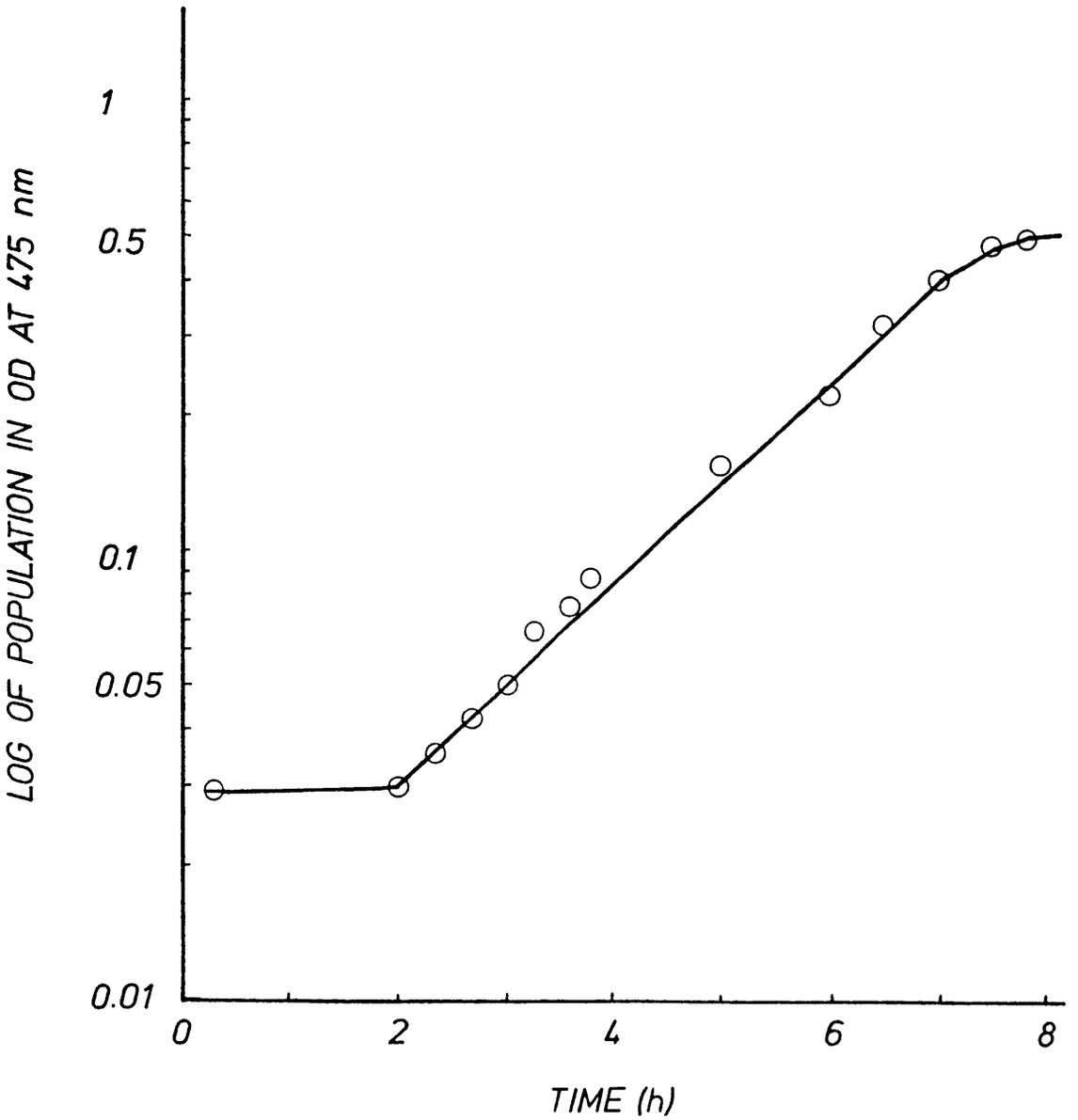

FIGURE 3--Growth curve of Bacteroides fragilis VPI 531. The cells were grown in pre-reduced PYG broth and incubated anaerobically at 37 C.

intervals of time. Irradiated cells were then propagated on PYG agar at 37 C for 24 h under anaerobic conditions. The number of viable cells were determined as they developed into visible colonies. The data are shown in Table 3 and are illustrated in Figure 4.

Graphic illustrations of the results (Figure 4) obtained indicate that the number of viable cells of the organism decreased with increasing exposure time. The dose response survival curve for B. fragilis cells irradiated with 0.33 C/Kg Cs-137 followed the exponential kinetics of the single target theory similar to that of Pseudomonas aeruginosa (Azghani and Fuerst 1982) and E. coli (Casarett 1968).

For genetic studies of bacteria and other microorganisms the isolation of mutants with stable chromosomal markers are necessary. Attempts were made to isolate B. fragilis strains resistant to antibiotics such as chloramphenicol, clindamycin, lincomycin, rifampicin and tetracycline. These antibiotics are commonly used in treatment of Bacteroides infections in man. Cs-137 gamma rays were utilized to induce mutations in the test bacteria. Irradiated cells were transferred to prereduced PYG broth and incubated anaerobically at 37 C for 6 to 8 h. Cells were then propagated on PYG agar

Table 3--Effect of different doses of Cs-137 on the survival of Bacteroides fragilis VPI 531.

Time of exposure	Total dose R	Total dose C/Kg	Ave # of cells/ml ± standard deviation	Cells surviving
min				
0	0	0	$2.9 \times 10^7 \pm 3.7 \times 10^6$	1.0×10^2
8	1.03×10^4	2.65	$4.2 \times 10^6 \pm 9.2 \times 10^5$	1.4×10^1
10	1.29×10^4	3.32	$3.0 \times 10^5 \pm 3.0 \times 10^4$	1.0×10^0
12	1.54×10^4	3.99	$3.0 \times 10^4 \pm 2.0 \times 10^3$	1.0×10^{-1}
16	2.06×10^4	5.32	$8.0 \times 10^2 \pm 1.1 \times 10^2$	2.7×10^{-3}
20	2.58×10^4	6.65	$7.3 \times 10^2 \pm 8.4 \times 10^1$	2.5×10^{-3}

The samples were obtained from a bacterial suspension in pre-reduced phosphate buffer and were exposed to 1.29×10^3 R/min of Cs-137. Irradiated and control samples were diluted and then plated in aliquots of 0.1 ml. Cells were allowed to grow anaerobically for 24 h at 37 C. The number of colonies was determined on a Quebec colony counter. (R), Roentgen; (C/Kg), Coulomb/Kilogram.

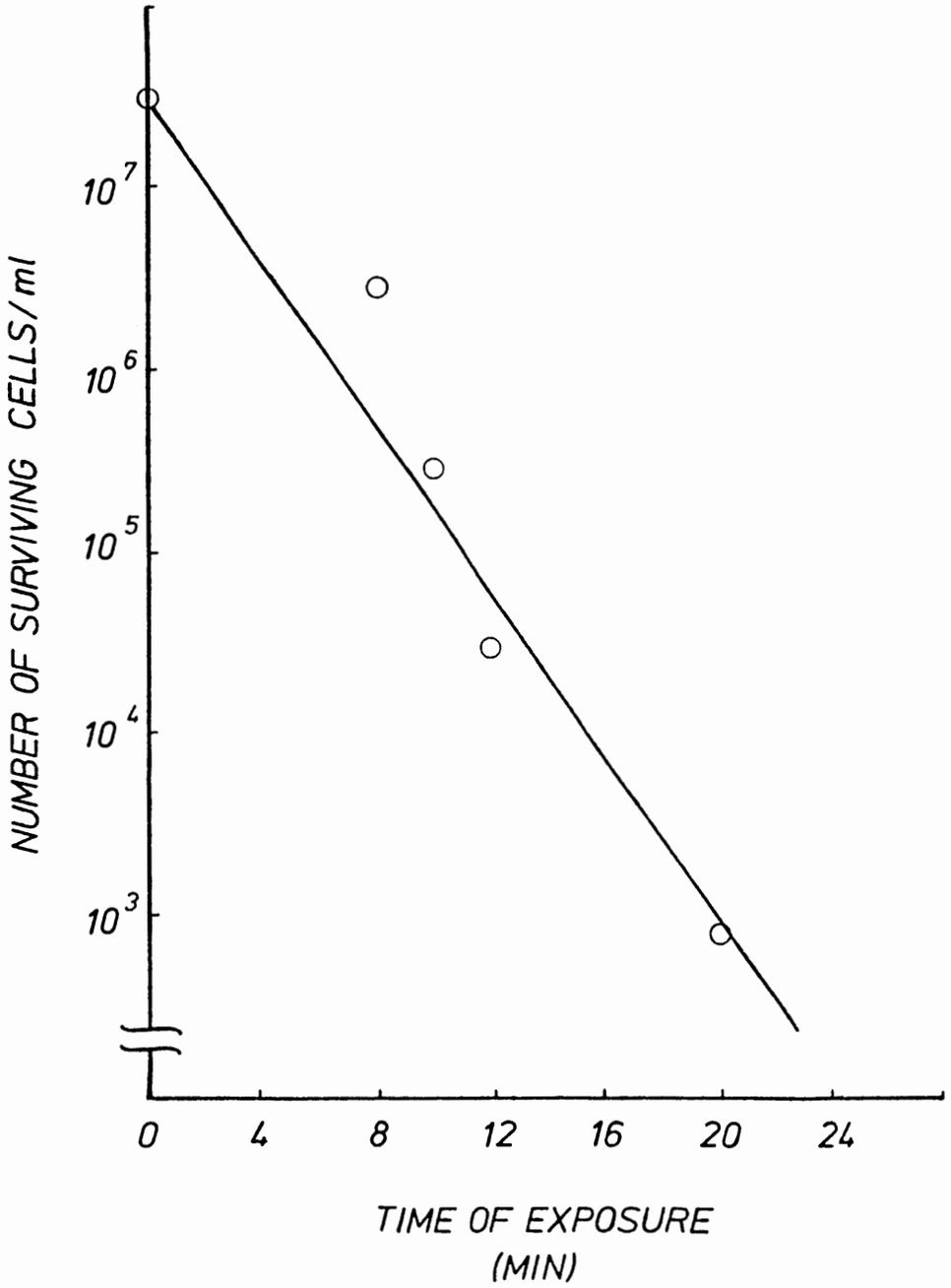

FIGURE 4--Graphic illustration of data indicating the effect of 1.29 x 10^3 R/min of Cs-137 on the survival of Bacteroides fragilis at various time intervals.

containing 10 µg/ml of the given antibiotics. Colonies grown on PYG agar with antibiotics were considered to be possible antibiotic resistant mutants.

After many trials, mutants of B. fragilis resistant to the antibiotics tetracycline and rifampicin were isolated. Tetracycline and rifampicin resistant mutants were maintained in chopped meat broth containing 10 µg/ml of the respective antibiotic. To confirm the identity of the mutants a series of biochemical and antibiotic sensitivity tests were performed along with observations of the appearance of the cells and their colony morphology. The data are shown in Table 4. The results indicate that tetracycline and rifampicin resistant mutants and the parent strain of B. fragilis share similar characteristics and except for the mutations are essentially the same strains.

Tetracycline and rifampicin resistant mutants of B. fragilis were also able to grow in a chemically defined medium, developed for isolation of auxotrophs of B. fragilis by Van Tassell and Wilkins (1978). This experiment indicated that the antibiotic resistant mutants of B. fragilis, that were isolated in the present study, did not lack the key enzymes which were involved in normal pathways for synthesis of compounds such as amino acids and/or nucleotides.

Table 4--Biochemical characteristics and antibiotic susceptibilities of tetracycline and rifampicin resistant mutants (tet^r, rif^r) of Bacteroides fragilis.

TESTS	REACTION	
	Tet^r	Rif^r
Fermentation of:		
Glucose	+	+
Esculin	-	-
Starch	+	+
Hydrolysis of starch	+	+
Production of indole	-	-
Sensitivity to:		
Clindamycin	s	s
Erythromycin	s	s
Amplicillin	r	r
Rifampicin	s	r
Tetracycline	r	r
Penicillin	r	r

The presence of acid in PY broth containing different carbohydrates was an indication of a positive reaction in the carbohydrate fermentation test Iodine was used to determine the hydrolysis of starch. Indole production was determined by the addition of xylene and Ehrlich's reagent in chopped meat broth cultures. Sensitivity and resistance were determined by the average size (mm) of the zone of inhibition around antibiotic disks. (+), positive or present; (-), negative or absent; (s), sensitive; (r), resistant.

Rifampicin is a hydrophobic antibiotic, which affects both Gram-positive and Gram-negative bacteria. The cellular target for rifampicin is DNA-dependent RNA polymerase (Hartmann et al. 1967; Wehrli et al. 1968). B. fragilis rifampicin resistant mutants obtained from irradiated populations were utilized to investigate whether RNA polymerase was a possible target site for rifampicin in B. fragilis. Crude cell extracts and partially purified enzymes obtained from parent strain and rifampicin mutants were examined for their in vitro RNA synthesis activities. RNA polymerase of E. coli (Sigma) was used as the control. It was found that a 10 min incubation of the enzymes with the assay mixture was sufficient to synthesize a measurable amount of RNA molecules in vitro.

Figure 5 indicates the in vitro inhibitory action of rifampicin on the incorporation of [^{3}H] ATP by RNA polymerase isolated from B. fragilis parent type and rifampicin resistant mutants. The radioactivity measurements for the enzymes extracted from rifampicin resistant mutants demonstrated a 50% reduction by 16 µg of rifampicin per ml of reaction mixture. However, only 6 µg of rifampicin per ml inhibited the incorporation of [^{3}H] ATP by 50%, when the enzyme from wild type B. fragilis was

Table 5--Minimal inhibitory concentrations (MICs) of different antibiotics tested against Bacteroides fragilis rifampicin resistant and wild type strains.

Antibiotics	MICs (μ/ml)	
	B. fragilis wild type	B. fragilis rif^r mutants
Rifampicin	0.39	100
Rifamycin	0.39	>100
Penicillin	>250	50
Tetracycline	1.56	3.12
Chloramphenicol	0.39	0.39

The MICs were determined according to the procedure on Table 2. The dilutions were set up in replicates of three for each antibiotic.

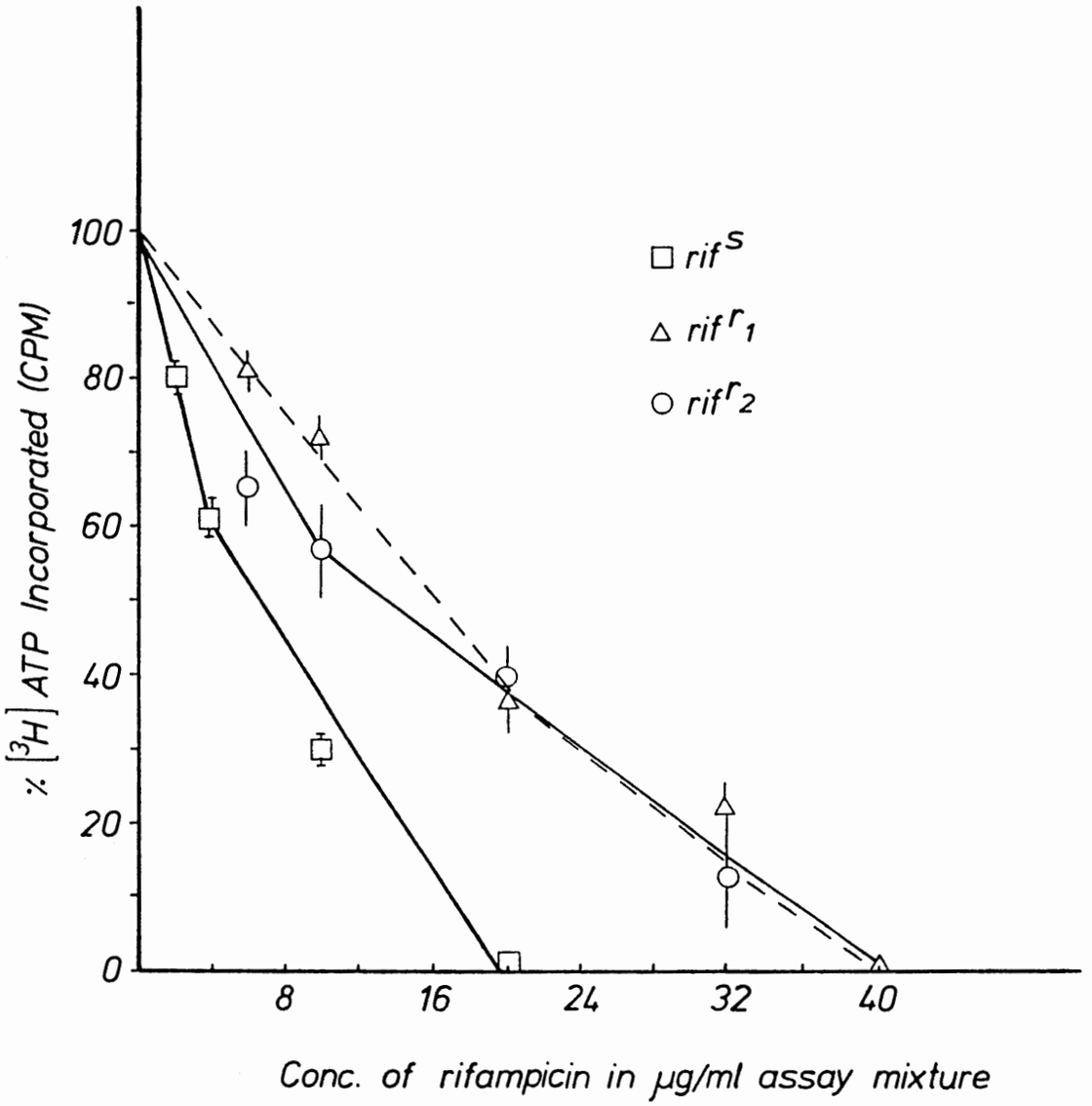

FIGURE 5--Effect of rifampicin on [^{3}H] ATP incorporation in Bacteroides fragilis RNA polymerase extracts, the rifampicin sensitive strain (rif^s), and the rifampicin resistant strains (rif^{r}_1, rif^{r}_2).

utilized. The difference in sensitivity to rifampicin between the enzymes isolated from normal and rifampicin resistant B. frigilis was therefore about threefold.

The sensitivity to various kinds of antibiotics of B. fragilis rifampicin resistant strains were compared to that of the parent strain. As shown in Table 5, higher concentrations of rifampicin were required to inhibit the growth of B. fragilis rifampicin resistant mutants. These strains were also resistant to rifamycin (a natural rifampicin) by a factor greater than 250. Increased sensitivities of the mutant strains to penicillin were detected. The MICs values of penicillin required to inhibit the growth of the mutant strains were found to be 50 µg/ml. A five times higher concentration of penicillin was required to achieve equal level of inhibition of the parent strain of B. fragilis. As may be seen in Table 5, the two strains responded similarly to inhibitory concentrations of chloramphenicol. However, a two fold higher concentration of tetracycline was required to inhibit the growth of rifampicin resistant strains.

B. fragilis VPI 531 produces acetic acid, formic acid, propionic acid and isovaleric acid as end-products of its volatile fatty acid metabolism. End-product analysis of the volatile acids, as determined by Gas

Liquid Chromatography, indicated that the mutant strains developed the same substances as those produced by the wild type strain. As shown in Figure 6 (courtesy of Mrs. Sheryl Kappus, Parkland Hospital, Dallas, TX), the volatile fatty acids formed as end-products of metabolism by the rifampicin resistant mutants were acetic, formic, propionic, and isovaleric acids, analogous to those of the parent strain.

Tetracycline is an antibiotic of choice in the treatment of anaerobic infections. Tetracyclines inhibit protein synthesis by interfering with aminoacyl transfer ribonucleic acid binding to the ribosomal A site in microorganisms (Bryan and Parr 1984). Given the fact that the ribosome is the target for tetracycline, it could be expected that alteration in binding of this antibiotic to ribosomes would be the mechanism of resistance of the mutants to this antibacterial chemotherapeutic agent. However, it has been shown that a decreased penetration of the drug into the cells is the mechanism for tetracycline resistance in E. coli (Okamoto and Mizuno, 1962).

A study was made of the uptake of tetracycline by the B. fragilis parent type and the tetracycline resistant strains. The later strains had been exposed to 3.99 C/Kg CS-137 gamma irradiation. Late log phase cells of both

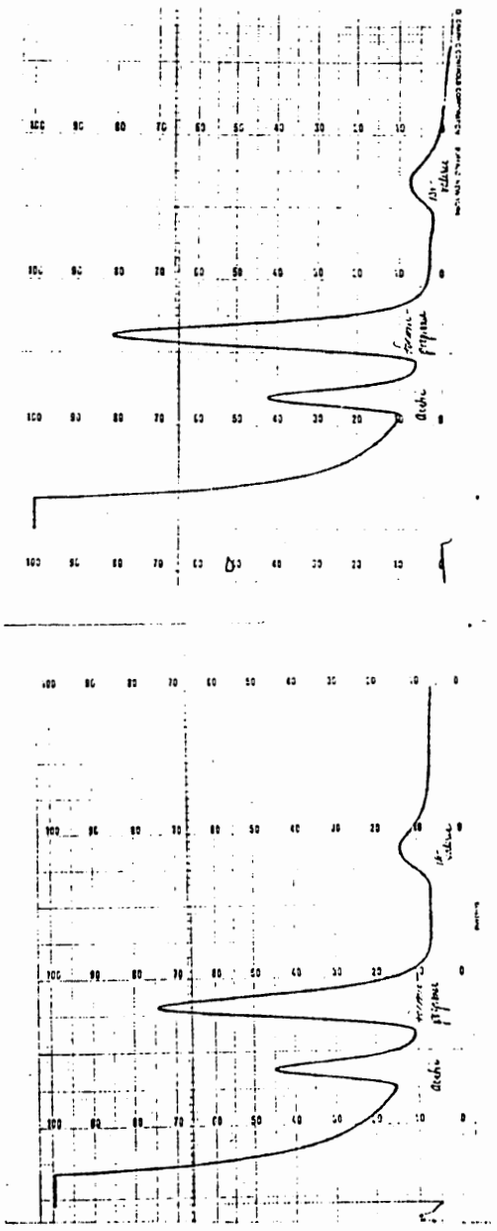

Figure 6--End-products analysis of volatile acids, produced by B. fragilis wild type and rif^r strains, determined by Gas Liquid chromatography.

strains were incubated with 1 µg/ml of [^{3}H] tetracycline with a total activity of 250 µCi for 3, 6, 30 and 60 min incubations at 37 C. Accumulations of labeled tetracycline by wild type and tetracycline resistant mutant cells were determined by liquid scintillation techniques (Beckman 9,000). Tetracycline sensitive strains of B. fragilis accumulated more [^{3}H] tetracycline than the mutant strains in any incubation time, as shown in Figure 7. However, statistical evaluation of the data as determined by the student's t test revealed that these differences were significant after 30 and 60 min of incubation time.

Figure 8 shows the accumulation of [^{3}H] tetracycline by B. fragilis normal, and tetracycline resistant strains, after 1 h incubation of the cells with labeled tetracycline. As Figure 8 illustrates, mutant strains did accumulate some tetracycline, however, student t test analysis indicated that they differed significantly from that accumulated by the parent type B. fragilis. In the second technique, introduced by Izaki et al. (1966), tetracycline sensitive and resistant strains of B. fragilis were grown in the presence of 0.5 µg/ml of the antibiotic for 3 h. Tetracycline uptake activity of the cells was then measured by incubating the cells in

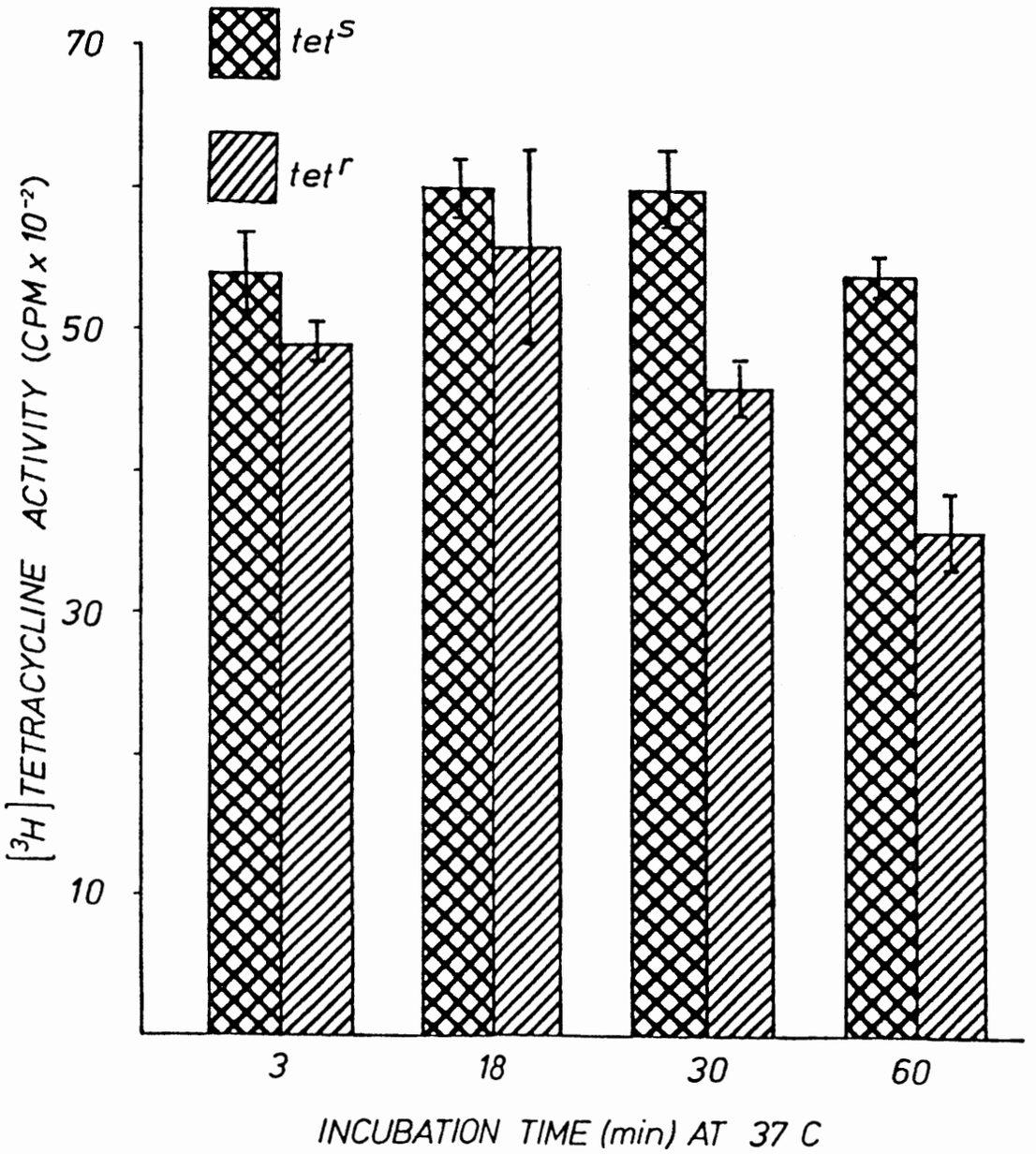

FIGURE 7-- Uptake of $[^3H]$ tetracycline by sensitive (tet^S) and resistant (tet^r) mutants of Bacteroides fragilis after the addition of 1 µg/ml of labeled tetracycline. The differences in uptake of tetracycline were significant after 30 and 60 min. of incubation time. The bars refer to standard deviation.

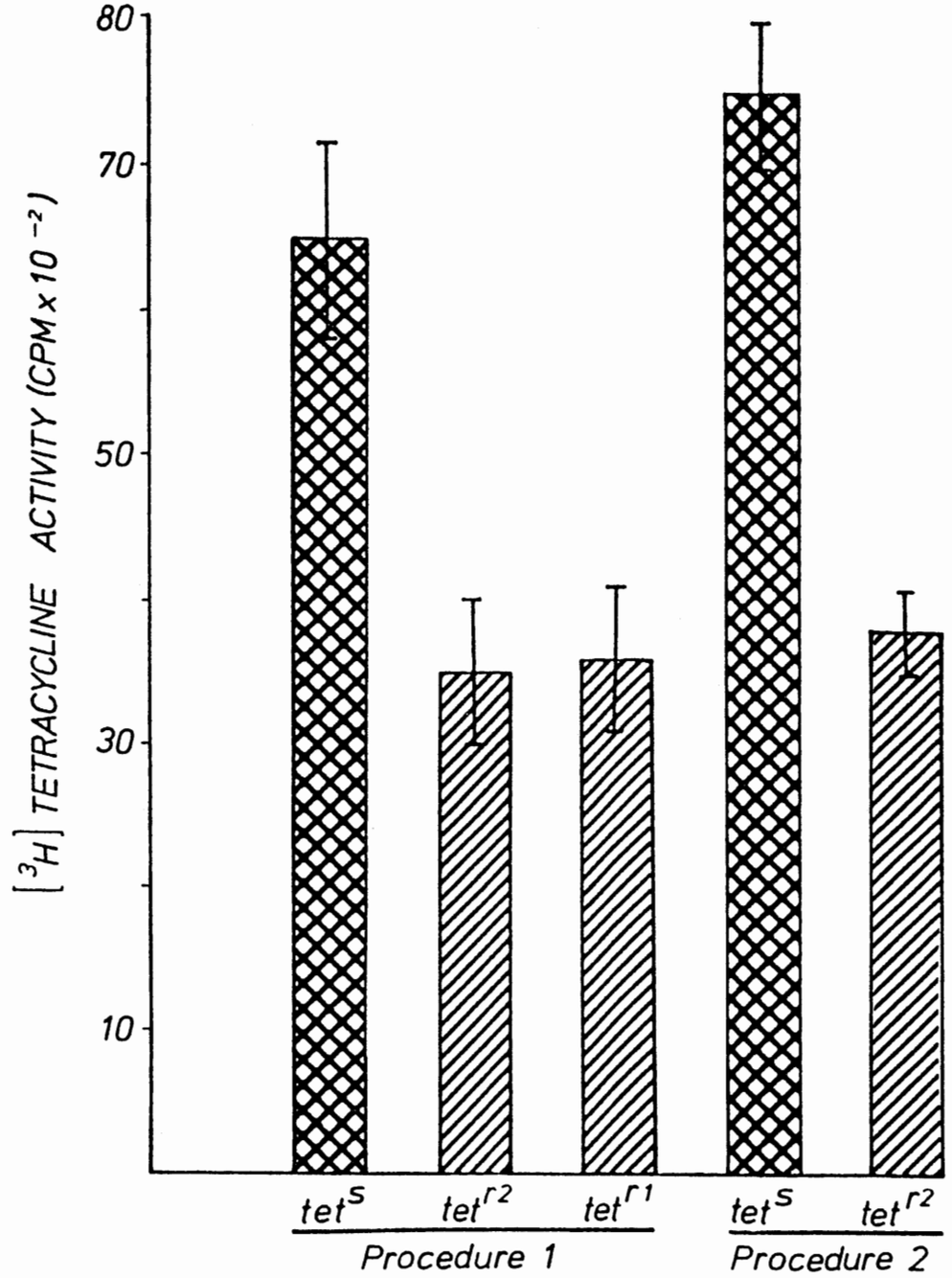

FIGURE 8-- Uptake of [^{3}H] tetracycline by sensitive and resistent mutants of Bacteroides fragilis after 60 min incubation at 37 C with 1 µg/ml of labeled tetracycline. The second procedure was done after preincubation of the strains with 0.5 µg/ml of cold tetracycline for 3 h. The bars indicate standard deviation.

Schaedler broth with 1 μg/ml of labeled antibiotic. This experiment was designed to test the effect of the presence of tetracycline on the ability of the cells to accumulate the antibiotic. As shown in Figure 8, similar results were obtained when the cells were grown in the presence or absence of tetracycline.

To overcome the increasing problem of bacterial resistance to tetracycline, derivatives of this antibiotic with more penetration ability such as minocycline and doxycycline have been developed (Kurytowicz 1976). These new antibiotics are more lipophilic than natural tetracyclines. As a result, they penetrate better across biological barriers (Kurytowicz 1976). Sensitivities of the two tetracycline resistant mutants of B. fragilis to minocyline and doxycycline were determined. it was found that tetracycline resistant B. fragilis strains were sensitive to the more lipophilic tetracycline derivatives, minocycline and doxycycline. As shown in Table 6 the MICs of doxycycline and minocycline were 16 times lower than those of tetracycline.

Table 6 indicates variations in MICs of antibiotics tested against the parent and tetracycline resistant mutants of B. fragilis. The mutants to tetracycline showed decreased sensitivity to doxycycline (two fold),

Table 6--Minimal inhibitory concentrations (MICs) of different antibiotics tested against the parent strain and tetracycline resistant variants of Bacteroides fragilis VPI 531.

Antibiotics	MICs (µ/ml)	
	B. fragilis tet^s	B. fragilis tet^r
Tetracycline	1.56	25
Doxyclin	0.78	1.56
Minocyclin	0.39	1.56
Penicillin	250	50
Rifampicin	0.39	3.12
Chloramphenicol	0.39	1.56

The MICs were determined according to the procedure illustrated on Table 2. The dilutions were set up in replicates of three for each antibiotic.

minocycline (four fold), rifampicin (eight fold), and chloramphenicol (four fold). However, these mutants became five times more sensitive to penicillin than their parent strain. Tetracycline mutants of B. fragilis, like rifampicin resistant strains, did not differ from the wild type in their biochemical activities such as fermentation of glucose, esculin and starch, hydrolysis of starch, or indole production (Table 4).

Most strains of B. fragilis isolated from clinical specimens are moderately or highly resistant to beta-lactam antibiotics (Dornbusch et al. 1975). This resistance seems to depend on the production of β-lactamases (Pinkus et al. 1968). Since the sensitivities of B. fragilis tetracycline and rifampicin resistant mutants differ from that of the parent strain, it was decided to determine whether these mutants could produce β-lactamase. A modified chromogenic cephalosporin assay described by Bourgault and Rosenblatt (1979), was utilized to test for β-lactamase production in these strains of B. fragilis. A prepared solution of nitrocefin was used. The compound is a cephalosporin, developed by O'Callaghan et al. (1972), which undergoes a distinctive color change when hydrolyzed by β-lactamases. It was found that the antibiotic resistant mutants of B. fragilis changed the

yellow color of nitrocefin to red within 15 min, indicating a positive reaction, although quantitative experiments would be required to compare the activity of the enzymes produced by these strains.

DISCUSSION

The results of the research reported in this dissertation pertain to effects of ionizing radiation on the survival of the anaerobic bacillus Bacteroides fragilis VPI 531. The mutagenicity of Cs-137 gamma irradiation was studied and antibiotic resistant mutants were isolated. These organisms were utilized to investigate the possible modes of action of tetracycline and rifampicin in B. fragilis.

Attempts were made to determine the effects of exposure to 1.29×10^3 R/min of Cs-137 gamma irradiation, for different intervals of time, on the survival of B. fragilis. Exposure to 2.06×10^4 R (5.32 C/Kg) induced an $LD_{99.99}$ in the Bacteroides population samples. This dose was obtained after 16 min irradiation with 7.75×10^4 R/h of Cs-137.

The literature does not disclose other investigations where B. fragilis was exposed to ionizing radiation sources. In studies with other bacteria, Azghani and Fuerst (1982) found that for the aerobic bacillus Pseudomonas aeruginosa a total dose of 1.40×10^4 R brought about an $LD_{99.99}$. This result is in agreement

with the data obtained by Lehrbach et al. (1976), who found that 16 Krads would kill the entire population of Pseudomonas aeruginosa.

The irradiation required to reduce the number of individuals in the population to 37% and used as a quantitative measure of radiation sensitivity, was calculated as 1.42 X 10^2 R for Ps. aeruginosa (Azghani and Fuerst 1982). The experimental results as reported earlier showed a higher D_{37} value for B. fragilis (2.58 X 10^2R), which suggested that this organism may be more resistant to gamma radiation than Ps. aeruginosa.

Damage to living organisms by ionizing radiation is generally enhanced by the presence of oxygen during exposure. Hodgkins and Alper (1963) reported that anaerobic growth of E. coli B after X radiation decreased lethal damage. Anaerobiosis was more effective after X rays were delivered in the absence rather than in the presence of oxygen. B. fragilis was exposed to Cs-137 in a pre-reduced phosphate buffer containing E. coli membrane fragments, which reduced the oxygen tension to about 5% in the medium. Hence, the difference observed in radiosensitivity between Ps. aeruginosa and B. fragilis could be, in part, due to the low tension of oxygen in the Bacteroides cell suspensions.

Evidence for membrane involvement in cellular radiosensitivity may be found in the literature. Reports by Femingo and Akoev (1979) showed that exposure of Streptococcus faecalis to gamma radiation resulted in an increased permeability for K^+ and protons, which was probably due to structural rearrangement in the membrane resulting from irradiation. Redpath and Patterson (1978) showed that differences in fatty acid composition of the cell membrane of E. coli were related to altered radiosensitivity of the organism. It may be speculated that the structure of the membrane is one reason for the high sensitivity of Ps. aeruginosa to ionizing radiation.

Bacteroides cell membranes have an unusual lipid composition. Approximately 50% of the lipids that are extractable with chloroform-methanol are sphingolipids or free ceramides (Salyers 1984; Miyagawa et al. 1979). Miyagawa et al. (1979) reported the occurrence of 3-hydroxy fatty acids as major components of the extractable lipids from B. fragilis. However, the fatty acids of phosphosphingolipids in Bdellovibrio bacteriovorus which is a Gram-negative strict aerobe, have been shown to be 2-hydroxy fatty acids (Steiner et al. 1973). Studies of the composition of Bacteroides lipopolysaccharides (LPS) have confirmed that they differ in several

aspects from LPS of other Gram-negative bacteria (Salyers 1984). For instance, Bacteroides LPS lacks 3 keto-2 deoxy-octanoic acid and heptose in its structure (Kasper et al. 1979). Whether the marked difference of fatty acid compositions in sphingolipids and LPS of Bacteroides had anything to do with the radiosensitivity of the test organism remains to be studied. However, these differences may reflect dissimilarities in the fatty acids and LPS synthesizing processes between anaerobes and aerobic bacteria (Miyagawa et al. 1979).

Three different types of DNA repair have been described in bacteria. Photoreactivation, an enzymatically controlled repair mechanism, eliminates UV radiation induced pyrimidine dimers. Dark repair, often referred to as excision repair, restores lesions induced by a variety of radiation and chemical mutagens. Finally, repair of damage caused by ionizing radiation is done by the recombination repair system. Most bacteria possess one or more of these repair pathways (Dertinger and Jung 1970; Kung and Lee 1973).

Confirmed evidence for the mechanism by which Bacteroides repairs ionizing radiation induced damage has not been cited in the literature. However, based on studies by Jones et al. (1980); Slade et al. (1981); and

Schumann et al. (1982), it has been suggested that unlike in the aerobic and facultive anaerobic bacteria, inactivation of B. fragilis by far-UV is oxygen dependent and that the induced repair systems in B. fragilis are not the same as those in E. coli.

Attempts were made to study mutagenic effects of ionizing radiation on B. fragilis VPI 531. Mutants resistant to rifampicin and tetracycline, were isolated. These isolates, resulting after insult with radiation and antibiotic treatment of the exposed cells, were obtained from the pre-reduced ABHI agar plates containing 10 µg/ml of the respective antibiotic. The irradiation experiments as performed did not result in the isolation of chloramphenicol, lincomycin, or clindamycin resistant mutants.

To isolate antibiotic resistant mutants, two different procedures were employed. In the so called direct technique, irradiated cells were diluted in warm (45 - 50 C) ABHI agar containing 10 µg/ml of the antibiotics, distributed on plates and incubated anaerobically at 37 C for 48 - 72 h. This method was designed to determine the mutation rate. Attempts to isolate antibiotic resistant mutants were unsuccessful when this technique was utilized.

Since B. fragilis is an obligate anaerobic bacterial organism it would take about 2 h to reduce the E_h of the

medium to a level that could be tolerated by the cells (Holdman et al. 1977). During this relatively long period of time very few mutants of the reduced number of surviving cells retained their viability; consequently, they were not isolated after 72 h of incubation required to grow Bacteroides. To overcome this problem, a second procedure attempting mutant isolation was utilized. Irradiation cells were propagated in ABHI broth for 6 - 8 h at 37 C and then subcultured in ABHI agar plates containing 10 µg/ml of the antibiotic. After several attempts, tetracycline and rifampicin resistant bacteria were isolated.

B. fragilis rifampicin resistant mutants were used to investigate the possible mode of action of this antibiotic on the test organism. The mechanisms by which tetracycline and rifampicin inhibit the growth of aerobic bacteria have been well established in the literature (Wehrli et al. 1968; McMurry and Levy 1978; McMurry et al. 1982). Rifampicins have been known to inhibit the activity of RNA-polymerase in E. coli and Staphylococcus aureus (Hartmann et al. 1967; Wehrli et al. 1968).

DNA dependent RNA-polymerases from sensitive and resistant strains of B. fragilis were isolated and partially purified. Activities of the enzymes were

determined in assay mixtures by percent incorporation of [^{3}H] ATP in the RNA molecules synthesized in vitro. Figure 5 shows the degree of inhibition of RNA polymerase isolated from the B. fragilis parent and rifampicin resistant mutant strains. Six µg rifampicin per ml of the reaction mixture inhibited the incorpation of [^{3}H] ATP by 50% in the enzyme extracted from the sensitive strain. In contrast, RNA-polymerase isolated from B. fragilis resistant to rifampicin, was inhibited by a concentration of 16 µg of antibiotic per ml reaction mixture. The difference in sensitivity to rifampicin of RNA-polymerase extracted from normal (rifampicin sensitive) and rifampicin resistant B. fragilis was therefore 2.5 fold.

Rifampicin resistant mutants have been isolated from different bacterial species, including E. coli (Tocchini-Valentini et al. 1968). The activity of RNA polymerase in cell free extracts has been analyzed by several investigators interested in the mechanism of rifampicin resistance in bacteria (Mindlin et al. 1972; Wehrli 1968). Mindlin et al. (1972) analyzed the response to rifampicin of RNA polymerase obtained from 60 spontaneous rifampicin mutants of E. coli. They demonstrated various levels of rifampicin resistance among the enzymes isolated from

different rifampicin mutants. The RNA polymerase region on the E. coli chromosome was found to be affected in all mutants having an RNA polymerase enzyme resistant to different levels of rifampicin.

The mutation to rifampicin resistance is known to be localized in the rpoB gene encoding the β subunit of RNA polymerase (Mindlin et al. 1972). Ovchinnikov et al. (1981) determined the primary structure of the rpoB gene cloned from rif^r mutants on the E. coli chromosome. Ovchinnikov et al. (1981) found that mutant genes contain an ATTA transversion, entailing the substitution of a valine residue into the mutant RNA polymerase for the aspartic acid residue of the normal β subunit.

Other experiments were performed by means of the tube dilution methods to determine minimal inhibitory concentrations of rifampicin on B. fragilis rifampicin resistant mutants. These strains were inhibited more by higher concentrations of rifampicin and rifamycin SV, a natural rifampicin, than the sensitive strains. The difference between MICs of the sensitive and resistant strains indicated that some other factor(s) were involved in the responses toward the antibiotics, such as alteration in permeability of the bacterial membrane.

Bacterial resistance to tetracycline is a very common finding, and its prevalence is now much greater than previously encountered (Levy 1984). Tetracycline resistant mutants were isolated after insult of B. fragilis with ionizing radiation and exposure to tetracycline. In further investigations, the mechanism by which B. fragilis became resistant to tetracycline was studied. Two different techniques were utilized to compare the uptake of tetracycline by parent and tetracycline resistant mutants of B. fragilis. The results of this experiment (Figure 8) show that the mutant strains take up less tetracycline during 1 h incubation with [^{3}H] tetracycline than the parent strain. This indicates that induced changes in membrane permeability could result in mutant strains that are resistant to tetracycline.

The outer membrane of Gram-negative bacteria is an unusual biological membrane in that its exterior monolayer includes LPS as its major lipid molecules, while the inner leaflet contains phospholipids rather than LPS. The outer membrane also contains proteins, some of which are involved in the formation of water-filled channels, called porins. Hydrophilic compounds can pass across the outer membrane via these porins (Norton 1981). The molecular properties and channel area of individual porins, and

therefore the effective exclusion limit of porins and of outer membranes, varies from organism to organism (Hancock 1984).

Studies on low-level tetracycline resistant E. coli cells have demonstrated a change in major outer membrane proteins or porins (Nikaido and Nakae, 1979). LPS also affects tetracycline activity. Leive et al. (1984) reported that mutations in the LPS of E. coli altered the sensitivity of these mutants to the more lipophilic tetracycline. These findings, along with the observations (Table 6) that MIC values of doxycycline and minocycline (lipophilic derivatives) were not altered significantly in tetracycline resistant strains, indicated that alterations in porins content of the outer membrane possibly decreased the rate of entry of tetracycline into the mutant cells.

In Gram-negative facultative anaerobes, plasmid mediated tetracycline resistance is generally an inducible property (Fayolle et al. 1980). Levy and McMurry (1978) identified a plasmid-encoded inner membrane protein, designated TET protein, involved in the transport of tetracycline. They showed that the synthesis of TET protein is negatively regulated. Reduced rapid uptake of tetracycline by induced mutants was the primary effect of tetracycline resistance induction. Furthermore, oxidation

phosphorylation inhibitors such as 2,4-dinitrophenol (DNP) induced the rapid uptake system in mutants, whereas it did not affect that of sensitive cells. In light of these findings, Levy and McMurry (1978) concluded that resistant cells may have an energy-dependent mechanism (but not ATP) for blocking the entry or stimulating efflux of the antibiotic. The slow-uptake system in induced resistant cells was not energy dependent, whereas in sensitive cells this uptake system was an active process. All these findings were indication of a different uptake system for induced resistance of E. coli to tetracycline.

B. fragilis VPI 531 was found to be resistant to penicillin (MIC 250 µg/ml). Weinrich and Del Bene (1976) have shown that some isolates of B. fragilis produce beta-lactamase, the enzyme that inactivates the β-lactame antibiotics. These antibiotics include: penicillins, cephalosporins, monobactams, and cephamycins. All β-lactame antibiotics are inhibitors of bacterial cell-wall synthesis (Bryan and Parr 1984). The MIC values of penicillin were determined for antibiotic resistant mutants of B. fragilis. The results of the data revealed a five fold increase in sensitivity of tetracycline and rifampicin resistant strains of B. fragilis to penicillin (Tables 5 and 6).

Beta-lactame antibiotic-hypersensitive mutants have been reported in E. coli, Pseudomonas aeruginosa, and Proteus vulgaris (Hamakado et al. 1985). Pleiotropic properties and the increased susceptibilities of these mutants to lysis by detergents such as SDS have been considered to be due to outer membrane alterations in these organisms (Hamakado et al. 1985). As discussed earlier, Gram-negative bacteria possess an outer membrane that acts as a barrier to the movement of hydrophobic and hydrophilic molecules into and out of the cell. Whether or not the changes in the lipids, lipopolysaccharides, and/or proteins of the outer membrane of B. fragilis antibiotic resistant mutants have been associated with increased sensitivity of these mutants to penicillin, remains to be investigated.

Experiments were performed to detect the production of β-lactamase enzyme by parent and antibiotic resistant mutant strains of B. fragilis. The production of beta-lactamase was determined by the chromogenic cephalosporin assay. In this experiment a 500 µg/ml solution of nitrocefin was used. This compound is one of a group of related cephalosporins which undergo a distinctive color change when hydrolysed by β-lactamase. Results of the experiments showed that the B. fragilis parent type and

the antibiotic resistant strains produced β-lactamase enzymes.

From the results obtained, it is suggested that ionizing radiation is a suitable mutagenic agent for anaerobic bacteria. However, the conservative nature of the B. fragilis genome, as well as technical difficulties would demand considerable efforts exerted in mutation studies of this anaerobe. Studies on tetracycline resistant mutants of B. fragilis isolated after irradiation, suggest that the resistance to this antibiotic is associated with the outer membrane permeability. The difference in inhibitory action of rifampicin on RNA polymerase activity, from rifampicin sensitive and resistant strains of B. fragilis, reveals that this enzyme is a possible suitable target for inhibition of bacterial growth in anaerobes by rifampicin. Changes in sensitivity to penicillin were observed in both tetracycline and rifampicin resistant strains of B. fragilis. Since the parent strains and the mutants produce β-lactamase, the enzyme that inactivates the β-lactame antibiotics, the increased sensitivity to penicillin of the mutant strains is speculated to be due to cell surface membrane alterations.

Certainly, the genome of B. fragilis, wild type and mutant strains, needs to be elucidated and mapped with techniques applicable to anaerobes using the technologies developed in studies with E. coli. Recombinant DNA methods and immunologic processes will be applied to understand outer membrane structure and function of bacterial anaerobes. In time chromosome maps of B. fragilis will be as familiar to the scientific investigator as the genetic structures of E. coli are now. With respect to the mutants obtained in the study presented here, changes in structure and function of proteins, lipids and lipopolysaccharides of the cell walls of these mutants remains to be investigated.

SUMMARY

1. The reports of high frequency of hospital infections attributed to Bacteroides fragilis, and of the increased resistance of this organism to commonly used antibiotics, stimulated the investigation reported in this dissertation. The genus Bacteroides is an obligate anaerobic bacillus normally found in the upper respiratory tract, the colon, and the genitourinary system. In the Bacteroides fragilis group, B. fragilis subsp. fragilis is the most important pathogen. It is frequently isolated from patients with soft tissue infections and constitutes the most common anaerobe that invades the blood stream.
2. The objective of the research reported in this dissertation was to isolate various mutants of B. fragilis VPl 53l, using gamma irradiation as the mutagenic agent. The resultant mutants would be utilized to reveal the mechanisms by which this group of organisms becomes resistant to select chemotherapeutic agents.
3. Modified pyrex Turner bulbs with a 100 ml liquid capacity containing 10 ml of cell suspensions in

pre-reduced phosphate buffer were employed for the exposure of the test organism to Cs-137 ionizing radiation. Escherichia coli B/r membrane fractions were utilized as a reducing agent in the medium. Gamma irradiation was applied to the bulbs at the rate of 7.75×10^4 R/h from a Mark I Cs-137 irradiator. A $LD_{99.99}$ of B. fragilis was obtained after 12 min of irradiation.

4. Attempts were made to isolate B. fragilis strains resistant to antibiotics such as chloramphenicol, clindamycin, lincomycin, rifampicin and tetracycline. Mutants of B. fragilis resistant to the antibiotics tetracycline and rifampicin were obtained.
5. B. fragilis rif^r mutants were utilized to investigate whether RNA polymerase was a possible target site for rifampicin in this anaerobe as is the case in E. coli. When the in vitro activity of RNA polymerase, isolated from rifampicin mutant strains, was compared with that of rifampicin sensitive B. fragilis, it was found that the difference in sensitivity to rifampicin between normal and rifampicin resistant B. fragilis was about three fold.
6. The uptake of tetracycline by mutants and parent strains of B. fragilis was compared. It was found

that tetracycline resistant mutants accumulated less tritiated labeled tetracycline than the normal strains, as determined by the liquid scintillation technique.

7. The sensitivity of the rifampicin and the tetracycline resistant mutants was not altered significantly toward more lipophilic derivatives of tetracycline (minocycline and doxycycline), rifamycin and other antibiotics tested. However, these mutants were more sensitive to penicillin than their parent strains.
8. The mutant strains of B. fragilis did not differ from the parent strain in their biochemical characteristics such as fermentation of glucose, esculin and starch, hydrolysis of starch, or indole production.
9. Attempts to isolate biochemical and antibiotic mutants, resistant to clindamycin, chloramphenicol, and lincomycin were unsuccessful. This is, in part, due to the conservative nature of the B. fragilis genome, resulting from its unique repair system.

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